

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 9/127, 39/00	A1	(11) International Publication Number: WO 93/14744 (43) International Publication Date: 5 August 1993 (05.08.93)
(21) International Application Number: PCT/US93/01102 (22) International Filing Date: 4 February 1993 (04.02.93) (30) Priority data: 07/830,612 4 February 1992 (04.02.92) US 07/885,905 18 May 1992 (18.05.92) US 07/942,639 9 September 1992 (09.09.92) US (71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US). (72) Inventors: BARCHFELD, Gail, L. ; 2225 Romey Lane, Hayward, CA 94541 (US). OTT, Gary ; 112 Marlow Drive, Oakland, CA 94605 (US). RALSTON, Robert ; 52 Long View Court, Danville, CA 94526 (US). SELBY, Mark ; 136 Galewood Circle, San Francisco, CA 94131 (US). SPAETE, Richard ; 2501 Coronet Blvd., Belmont, CA 94002 (US). VAN NEST, Gary ; 4890 San Pablo Dam Road, El Sobrante, CA 94803 (US). WALKER, Christopher ; 434 San Marin Drive, Novato, CA 94945 (US).		(74) Agents: McCLUNG, Barbara, G. et al.; Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608-2916 (US). (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: NON-TOXIC LIPID VACCINE FORMULATIONS (57) Abstract Suspensions of cell-membrane fusible lipids are capable of delivering antigens directly to the cytoplasm of host cells, bypassing the normal endocytic pathway. This results in more efficient presentation of the antigen in the context of the host cell class I MHC protein, which in turn provides class I MHC-restricted cytotoxic T cell immunity.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	MI	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

5

NON-TOXIC LIPID VACCINE FORMULATIONS

10

Description

15

Technical Field

This invention relates generally to the fields of immunology and vaccine formulation. More specifically, the invention relates to the use of cationic lipids in vaccine formulations for induction of class I MHC-restricted T lymphocyte immune responses in mammals and birds.

25

Background of the Invention

Protective humoral and cellular immune responses against a variety of pathogens can be elicited by vaccination with protein antigens. Humoral immune responses are antibody-mediated, while T lymphocytes are the effectors of cellular immunity.

5 The two types of T lymphocytes that can be distinguished based on function and surface markers are helper (T_H) and cytotoxic (T_C) cells. T_H cells express the CD4 surface protein and upon contact with foreign antigen, activate antibody-producing B cells and T_C . T_C express the CD8 protein, and are thought to control infection by elimination of cells expressing foreign antigens. Both T_C and T_H lymphocytes recognize antigens as
10 short peptides that are bound to cell surface class I and class II major histocompatibility complex (MHC) molecules, respectively.

 The mechanism by which antigens are processed into peptides differs for class I and class II MHC presentation. Antigens taken into the cell via endocytosis are digested into peptides that then bind to class II, but not class I, MHC molecules. In con-
15 trast, antigens that are synthesized within the cytoplasm or endoplasmic reticulum selectively bind to class I MHC molecules. Thus, vaccines containing subunit antigens, which gain access to only the endocytic pathway of the cell, prime the MHC class II restricted T_H but not class I restricted T_C . Priming of the T_C subset with non-replicating antigens will require delivery vehicles that bypass the endocytic uptake pathway and deliver vac-
20 cine proteins directly to the cytoplasm of antigen presenting cells. Many commonly used adjuvants such as water and oil emulsions (*i.e.* complete Freund's adjuvant) and aluminum hydroxide (*i.e.* alum) are not capable of directing antigens to cytoplasm of cells, and thus are not capable of priming the T_C subset. One possible solution to this obstacle is the use of antigen-containing liposomes or lipids that can bind to and fuse with cell membranes.

25 Liposomes are small vesicles formed from lipid bilayers, and may be prepared in a variety of different types. Different forms of liposomes are customarily designated by three-letter abbreviations. Thus, SUVs are small unilamellar vesicles, LUVs are large unilamellar vesicles, and MLV are multi-lamellar vesicles. Other abbreviations

are coined by their inventors, such as REVs for reverse-phase evaporation vesicles. Unilamellar vesicles have only a single lipid bilayer enclosing an aqueous volume. Multilamellar vesicles have a series of lipid bilayers arranged like the layers of an onion, with a central aqueous volume and additional aqueous layers trapped between the concentric lamellae. Liposomes have been used to deliver pharmaceuticals, either encapsulating a sensitive compound within the relatively protected aqueous volume, or to solubilize and deliver lipophilic compounds embedded within the bilayer. Liposomes in the bloodstream are generally taken up by the liver and spleen, and by macrophages. Thus, liposome formulations tend to target the formulated compound toward those tissues and cells. Fortunately, macrophages are one of the primary APCs.

N.A. Latif *et al.*, Immunol Lett (1987) 15:45-51 described the use of neutral and positively-charged liposomes (using stearylamine) to immunize rabbits with lysozyme. Latif administered lysozyme in several forms: free, encapsulated in neutral or cationic liposomes, and conjugated to the surface of neutral or cationic liposomes. These formulations were also administered with or without complete Freund's adjuvant. The formulations in which lysozyme was encapsulated in cationic liposomes provided the highest antibody titers.

E.K. Barbour *et al.*, Vet Immunol Immunopath (1989) 22:135-44 and Vet Immunol Immunopath (1990) 26:115-23 reported vaccination of chickens using *Mycoplasma gallisepticum* in positively charged multilamellar liposomes. Results were reported in terms of antibody titers, and "protection" based on the number of eggs laid. Antigen administered in liposomes was equivalent to antigen administered in oil emulsion.

C. Audera *et al.*, Clin Exp Allergy (1991) 21:139-44 disclosed the use of neutral, anionic, and cationic liposomes to administer allergens as a mouse model for allergy immunotherapy. Cationic liposomes stimulated the highest IgG response, while neutral and anionic liposomes stimulated the highest IgE response.

B. Frisch *et al.*, Eur J Immunol (1991) 21:185-93 studied the administration of peptides using liposomes. Frisch *et al.* noted that anionic liposomes are reportedly more trophic for macrophages, but failed to find any charge dependence in their results.

Abai *et al.*, WO 90/14074 disclosed liposomal formulations for delivering
5 therapeutic nucleotide analogs parenterally.

Felgner *et al.*, WO90/11092 disclosed a method for effecting vaccination by transfecting muscle cells with a polynucleotide encoding an antigenic protein using a cationic lipid. In one of Felgner's preferred embodiments, a small amount of polymerase is included with the polynucleotide to provide for translation of the polynucleotide immediately upon transfection. Cells thus transfected may express the encoded polypeptide during the lifetime of the polynucleotide, potentially for the entire life of the cell. However, this approach has inherent drawbacks. Exogenous polynucleotides (particularly RNA) are subject to degradation following introduction into a host cell. Also, it can be difficult to control the copy number of plasmids, which may result in expression of the heterologous polypeptide at toxic levels. Additionally, it can be difficult to insure expression, particularly in *in vivo* transfection where it is unacceptable to select against non-transformed or non-expressing cells. There is also the possibility that transfection with DNA encoding a viral envelope protein could result in "rescue" of defective viruses. For example, immunization with DNA encoding the hepatitis B virus surface antigen (HBsAg) could activate a latent hepatitis delta virus infection. In addition, *in vivo* expression of oncogene products (oncoproteins) in order to raise or augment a tumor-specific cytotoxic immune response against such oncoproteins would be unacceptable because of the potential for integration of the transfected oncogene into the host genome. Use of viral vectors such as vaccinia for *in vivo* expression of oncoproteins, could also be unacceptable.

25 C.R. Alving, J Immunol Meth (1991) 140:1-13 reviewed the current state of the art with regard to the use of liposomes in vaccine formulations. Alving noted that liposomal formulations have been demonstrated to effectively induce humoral (antibody-mediated) immunity, and are sometimes able to induce cell-mediated immunity. The lipid

composition, manner of preparation, surface charge, and other physical variables may all affect the effectiveness of the product formulation.

However, the form of immunity induced is not always the form desired. Most current formulations induce primarily humoral (antibody-mediated) immunity rather than cell-mediated immunity. Pathogens which replicate primarily intracellularly are often difficult to eradicate through humoral immunity, and are generally more susceptible to attack by cytotoxic T lymphocytes (CTL or T_C).

Disclosure of the Invention

One aspect of the invention is a composition for inducing (or enhancing) class I MHC-restricted T cell immunity in a mammal or bird, comprising a pathogen antigen in combination with a lipid formulation comprising a cell membrane-fusible positively charged lipid.

Another aspect of the invention is a method for inducing (or enhancing) class I MHC-restricted T cell immunity by administering to a mammal or bird in need thereof a composition comprising a pathogen or tumor antigen in combination with a lipid formulation comprising a cell membrane-fusible positively charged lipid.

Another aspect of the invention is a composition for administering non-nucleotidic compounds to the cytoplasm of a target cell, comprising a non-nucleotidic compound in combination with a lipid formulation comprising a cell membrane-fusible positively charged lipid.

Another aspect of the invention is a method of administering non-nucleotidic compounds to the cytoplasm of a target cell, comprising contacting the target cell with a non-nucleotidic compound in combination with a lipid formulation comprising a cell membrane-fusible positively charged lipid.

Modes of Carrying Out The Invention

A. Definitions

The term "cell membrane-fusible positively charged lipid" refers to a lipid which is positively charged under physiologic conditions, and is capable of spontaneous fusion with a cell membrane. Cell membrane-fusible positively-charged lipids may also be capable of forming stable liposomes without the addition of other lipids. In general, these lipids will comprise a positively-charged "head" and two lipophilic "tails." The tails are typically long (*e.g.*, C16-C32) linear alkyl, alkenyl, or alkynyl hydrocarbons. The head is typically a quaternary amine, substituted with three lower alkyl (*e.g.*, C1-C6) groups. Typical cell membrane-fusible positively charged lipids fall within the general formula $R_4\text{-CH(OR}_5\text{)-CH(OR}_3\text{)-N}^{\oplus}\text{(R}_1\text{)(R}_2\text{)(R}_3\text{) X}^{\ominus}$, where R_1 , R_2 , R_3 , and R_4 are each independently lower alkyl, R_5 and R_6 are each independently alkyl, alkenyl, alkynyl, or acyl of 12-32 carbons; and X^{\ominus} is a non-toxic counterion. Presently preferred cell membrane-fusible positively charged lipids are DOTAP and DOTMA. However, certain other lipids having positively-charged head and two lipophilic tails are also useful in the present invention, such as Transfectam (available from Promega). One may easily determine whether or not a lipid is a cell membrane-fusible positively charged lipid by repeating the experiments described in the Examples below, or by other techniques routine in the art.

The term "lipid suspension" refers to suspension of lipids or liposomes in aqueous solutions, and to oil-in-water emulsions formed from lipids.

The term "DOTAP" as used herein refers to the compound N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium methylsulfate. DOTAP is commercially available from Boehringer Mannheim, or may be prepared following the methods described by L. Stamatatos *et al.*, Biochem (1988) 27:3917-25; H. Eibl *et al.*, Biophys Chem (1979) 10:261-71.

The term "DOTMA" refers to the compound N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, which is commercially available under the name

Lipofectin® (available from BRL, Gaithersburg, MD), and is described by P.L. Felgner *et al*, Proc Nat Acad Sci USA (1987) 84:7413-17.

5 The term "alkyl" as used herein refers to radicals containing only carbon and hydrogen, and lacking double or triple bonds. Alkyl radicals within the scope of the present invention generally contain from 1 to 32 carbon atoms. "Lower alkyl" indicates alkyl radicals having from 1 to 6 carbon atoms, for example, methyl, ethyl, propyl, hexyl, and the like. Larger alkyl radicals are employed in lipophilic tails, such as C16, C18, C20, and the like.

10 The term "alkenyl" refers to radicals containing only carbon and hydrogen, having at least one double bonds, but no triple bonds. Alkenyl radicals within the scope of the present invention generally contain from 2 to 32 carbon atoms. "Lower alkenyl" indicates alkenyl radicals having from 2 to 6 carbon atoms, for example, ethenyl, propenyl, hexenyl, and the like. Larger alkyl radicals are employed in lipophilic tails, such as C16, C18, C20, and the like.

15 The term "alkynyl" refers to radicals containing only carbon and hydrogen, having at least one triple bond. Alkynyl radicals within the scope of the present invention generally contain from 2 to 32 carbon atoms. "Lower alkynyl" indicates alkynyl radicals having from 2 to 6 carbon atoms, for example, ethynyl, propynyl, hexynyl, and the like. Larger alkyl radicals are employed in lipophilic tails, such as C16, C18, C20, and the like.
20

The term "acyl" as used herein refers to a radical of the form $R-C(=O)-$, where R is alkyl, alkenyl, or alkynyl as defined above.

25 The term "antigen" as used herein refers to a molecule which is capable of immunoreactivity with an appropriate T cell antigen receptor. Antigens may comprise proteins, protein fragments, peptides, carbohydrates, lipids, and other molecules, but for the purposes of the present invention are most commonly proteins, short oligopeptides, oligopeptide mimics (*i.e.*, organic compounds which mimic the T cell antigen receptor-binding properties of authentic immunogenic peptides), oligosaccharides, or combinations

thereof. Suitable oligopeptide mimics are described, *inter alia*, in PCT application US91/04282. To specifically enhance a T_C cell response, in addition to a T_H cell response, it is preferred that the antigens be nonglycosylated or at least partially nonglycosylated, preferably, less than 50% carbohydrate by weight. For example, a preferred yeast-produced gp120 antigen ("env 2-3") is nonglycosylated and lacks a native secondary structure, whereas a mammalian (CHO) cell gp120 is 60% carbohydrate by weight. See e.g., Steimer, et al., Science 254:105-108 (1991); PCT Publ. No. WO 91/13906, published 19 September 1991. Glycosylation can be varied depending on the heterologous host (e.g., bacteria, baculovirus, yeast or mammalian express systems) used to recombinantly express said antigens; additionally the host can be treated with antibiotics to reduce glycosylation. Furthermore, the glycosylation of an antigen itself can be modified through the use of enzymes such as glucosidases, mannosidases and the like. Such methods are known in the art. When a T_C response is desired, the location of T_C epitopes in relation to glycosylation sites can be assessed so that the necessary T_C epitopes are available for an immune response. A "whole" vaccine contains whole virus or bacteria, either in attenuated form or as a killed or fixed preparation. A "subunit" vaccine is a vaccine in which the entire organism is not present, and typically contains one or two antigenic proteins derived from the pathogen. For example, Recombivax® (Merck, Sharp & Dohme) is a subunit vaccine for Hepatitis B virus which contains particles consisting of only the surface antigen (HBsAg).

The term "epitope" refers to the portion of an antigen which is immuno-reactive with a T cell antigen receptor. T cell epitopes are most commonly short oligopeptides, or organic mimics thereof. Most proteins and glycoproteins will exhibit a number of distinct and overlapping epitopes.

The term "viral structural protein" refers to a protein which plays a structural role in a virion or viral particle. Viral structural proteins are frequently envelope or core antigens, for example, HBsAg, HBcAg, HIV gp120, HIV gp41, HSV gB, HSV gD, *Chlamydia trachomatis* Major Outer Membrane Protein (MOMP), influenza hemagglut-

inin, and the like. "Viral structural protein" does not include those proteins which play solely non-structural roles, including typical transcriptional regulators, polymerases, toxins, and the like. It should be noted that antibody-mediated immunity is effective only against viral structural proteins, and in some cases pathogen-produced toxins (*e.g.*, diphtheria toxin). However, MHC-restricted cell-mediated immunity may be effective against even viral regulatory proteins and enzymes. The regulatory proteins and enzymes are not subject to the degree of antigenic variability that characterizes the structural (particularly envelope) proteins. Thus, compositions of the invention should be better to induce immunity to a wide variety of strains of any given pathogen. Further, the regulatory proteins and enzymes are less likely to under "antigenic shift", and thus are less likely to avoid immune surveillance.

The term "microbial structural protein" refers to a protein which plays a structural role in the surface structure of a pathogenic microorganism, typically a bacterium, plasmodium, yeast, or the like. Cell-mediated immunity is important for defense against intracellular infection by organisms such as *Mycobacterium leprae*, *M. tuberculosis*, *Plasmodium falciparum*, *P. vivax*, *C. trachomatis*, and the like. "Pathogenic microorganism" as used herein refers only to those microorganisms which cause or potentially cause disease or pathological symptoms in mammals.

The term "treatment" as used herein refers to (a) prophylaxis, or prevention of the disease state, (b) alleviation of symptoms or prolongation of remission periods, or (c) elimination of an existing disease state.

B. General Method

The first step in the practice of the invention is the selection of a suitable cell membrane-fusible positively charged lipid. Candidate lipids may be screened for suitability by repeating the simple assay described in Example 1. Alternatively, one may simply contact suitable target cells *in vitro* with a formulation comprising the candidate lipid in combination with an indicator molecule normally excluded by the cell membrane. One

may use a pH-indicating dye as the indicator molecule in order to distinguish membrane fusion events from simple endocytosis by the target cell (the target cell endosomes should register an acidic environment). Preferred cell membrane-fusible positively charged lipids will generally have a relatively small, positively-charged hydrophilic "head", such as a tri-alkyl quaternary amino group, and two, long hydrophobic "tails." Formulations may be obtained in prepared form (*e.g.*, Lipofectin is sold as a prepared suspension), or may be manufactured by standard techniques for the formation of liposomes or emulsions. Liposomes are typically prepared by hydration of a lipid film dried on the side of a glass vessel, by sonication of lipids in an aqueous suspension, by extrusion through a microporous membrane, by reverse-phase evaporation, and other techniques. A number of commercially available devices may be used to quickly prepare suitable formulations, such as the Liposomat® and the Microfluidizer® (Microfluidics Corp.).

An antigen is selected on the basis of the disease to be treated. As the present invention is designed to induce class I MHC-restricted T cell immunity, the antigens are selected based on the presence of protective epitopes. Whole subunit antigens are presently preferred, for example HSV gB and gD, because they are likely to contain both T-cell and B-cell epitopes. However, it is possible to use short epitopic peptides with the compositions of the invention as they are introduced directly into the cytoplasm of presenting cells, and thus may be made available for class I MHC presentation without proteolytic processing. This may prove particularly advantageous for immunization of individuals who exhibit unusual proteolytic enzymes or who lack some or all of the appropriate enzymes.

Antigens may be selected using standard methods. For example, a panel of candidate antigens may be screened with immune sera obtained from recovered or convalescent patients in order to determine which antigens contain immunodominant epitopes. T-cell haptens may be screened by exposing patient's peripheral blood lymphocytes (PBLs) to autologous cells incubated with the hapten. The haptens bind to surface MHC proteins: immune T-cells kill the presenting cells upon recognition of the hapten-MHC

complex. Systematic techniques for identifying B-cell and T-cell epitopes and their mimics have been described by H.M. Geysen, U.S. Pat. No. 4,708,871. Pathogens which may be suitably treated using the method of the invention include HAV (hepatitis A virus), HBV (hepatitis B virus), HCV (hepatitis C virus), HPV (human papilloma virus), HSV (herpes simplex virus), HIV (human immunodeficiency virus), SIV (simian immunodeficiency virus), EBV (Epstein-Barr virus), CMV (cytomegalovirus), poliovirus, meningococcus (A,B,C), *Helicobacter pylori*, cholera, varicella zoster virus (VZV), Dengvenins pertussis, diphtheria, tetanus, haemophilus influenza type b, measles, mumps, rubella, influenza, feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), enteroviruses, rotaviruses, and the like.

For treatment of cancer, one first identifies a tumor-specific antigen which is associated with class I MHC molecules. For these experiments, one employs human tumor cells and normal cells from which the tumor was derived, both from the same individual. Alternatively, certain tumor types have been identified which contain characteristic genetic alterations, specifically in oncogenes (*e.g. ras* in bladder and pancreatic cancer, HER-2 in breast cancer, *myc* in lung cancer) or in tumor suppressor genes (*e.g. p53* in colon cancer, pRB in retinoblastoma, *etc.*). One may create changes in gene expression in the laboratory by transferring one of these mutated or over-expressed genes into normal human cells of the appropriate type. For example, colon cancer cells frequently show mutations in p53. In order to model the effects of mutant p53 on gene expression in colon epithelial cells, one may obtain normal colon epithelial cells and introduce the mutant p53. The method of introduction may be transfection of a DNA construct which can express the mutant p53, infection with a virus which can express the mutant p53 (*e.g.,* amphotropic retrovirus, vaccinia virus, adenovirus, *etc.*), or by introduction of recombinant p53 protein using a formulation of the invention.

Having obtained the cancer cells and corresponding normal cells, changes in gene expression may be identified using standard techniques of molecular biology (*e.g.* subtractive hybridization of tumor cell RNA using normal cell cDNA, 2D electrophoresis

of proteins, followed by elution and sequencing of novel polypeptides, preparation of tumor-specific monoclonal antibodies for purification of tumor-specific proteins or identification of cDNA clones encoding these proteins from expression libraries. Another (preferred) method is to obtain peptides directly from Class I antigens of the cancer cells and corresponding normal cells and to compare these peptides by HPLC (*See e.g.* Grada *et al.*, Nature (1990) 348:213-16; Falk *et al.*, Nature (1990) 348:248-50; Rotzsche *et al.*, Nature (1990) 348:252-54). The sequence of these peptides, and the proteins from which the peptides originated may be identified or cloned using standard techniques.

The compositions of the invention are prepared simply by mixing the lipid formulation (liposome or emulsion) with the antigen, and permitting the mixture to incubate. The incubation period may range from 1 minute to overnight: presently, incubation for about 10 minutes is preferred. The amount of antigen used will in general depend upon the particular antigen selected and the condition to be treated. For example, for prophylaxis against HSV infection, one could administer from about 1 μ g to about 1000 μ g of recombinant HSV gD antigen to a human subject, more preferably about 10 μ g to about 100 μ g. For therapeutic treatment of HSV infection, one could administer from about 1 μ g to about 1000 μ g of recombinant HSV gD and gB antigens to a human, as a mixture. The dosages of other antigens and the use for treatment of other diseases will vary according to the immunogenicity of the antigens, the subject to be treated, the general health of the subject, the degree of infection present, and similar factors. Thus, one cannot specify a precise dose in advance. However, determination of appropriate dose ranges for any antigen and disease is within the level of ordinary skill in the art.

The composition is typically administered by parenteral means, such as by subcutaneous or intramuscular injection, or intravenous infusion. However, compositions of the invention are also suitable for administration by aerosol to the mucosa, for induction of mucosal immunity, *e.g.*, in the nose and sinuses, vagina, and other membranes.

The amount of lipid required will also vary somewhat with the type of antigen, depending on its degree of association with the lipid particles in the formulation.

This amount may vary with the electrostatic charge on the antigens and lipids, and their relative lipophilicities. However, as non-toxic lipids are preferably used, there is no critical upper concentration limit for the lipid component. In general, the concentration of lipid used should be between the minimum amount needed to deliver the antigen, and the maximum amount which can be formed into liposomes or emulsions. Most lipophilic and amphipathic antigens (*e.g.*, membrane-bound and membrane-associated antigens) can be delivered with about 0.1 to about 10 μg lipid per 1 μg antigen. When using commercial preparations of DOTAP, it is presently preferred to combine about 30 μL of DOTAP with about 70 μL of phosphate-buffered saline (PBS) to deliver about 100 μg of antigen such as HSV gB. Less lipophilic antigens may require slightly higher concentrations of lipid. Similarly, antigens which carry a positive charge may not associate well with the positively-charged lipids, and may require treatment to improve delivery. For example, positively charged antigens may be complexed with strongly anionic species to provide a net negatively-charged antigen complex, which then associates readily with the positively-charged lipid particles.

The compositions of the invention may also include factors to enhance expression of class I and class II molecules. For example, the vaccine preparation may contain gamma-interferon (γIFN), or transcription factors which up-regulate class I or class II expression. Because the formulations of the invention allow these proteins to enter the cell, class I expression may be activated in cells which lack receptors for γIFN . Various oncogenes (*e.g.* *N-myc*) and virus proteins (*e.g.* adenovirus E1a) have been shown to repress expression of class I antigens. The repressive effect could be overcome by addition of antisense RNA, blocking antibodies, and either class I proteins (with $\beta 2$ microglobulin) or DNA constructs which express class I MHC and $\beta 2$ -M from heterologous promoters which are not repressed by the oncogene products or viral proteins. This approach would be particularly useful for anti-tumor vaccine strategies which involve enhancing the immunogenicity of tumor cells. This approach previously has used recombinant retroviruses to deliver lymphokine genes (*e.g.*, IL2, TNF, M-CSF) into tumor

cells, followed by immunization of the patient (or experimental animal) with his own tumor cells (*e.g.*, Fearon *et al.*, Cell (1990) 60:397-403). These lymphokines may be delivered to tumor cells *in vivo* by formulations of the invention, eliminating the need for a recombinant retrovirus and allowing the lymphokines to be delivered along with tumor antigens.

Furthermore, to enhance a T_C cell response, in addition to a T_H cell response, it is preferred that the antigen of choice be nonglycosylated or at least partially nonglycosylated as discussed above. Furthermore, both the claimed compositions and submicron emulsion adjuvants can be used to enhance T_C cell response. "Submicron emulsion adjuvants" as used herein refer to those adjuvants described in PCT Publ. No. WO 90/14837, published 13 December 1990, wherein a submicron oil-in-water emulsion is described (optionally including a separate immunostimulating agent, *e.g.*, a muramyl peptide). In the examples below, this submicron emulsion adjuvant is designated "MF59".)

15 C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(Presentation In Vitro)

(A) The ability of DOTAP to deliver recombinant gB2 (see, e.g., PCT Publ. No. WO 88/02634, published 21 April 1988) to target cells for recognition by HSV-specific cytotoxic T lymphocytes was assessed. DOTAP (30 μ L) was mixed with 70 μ L of phosphate buffered saline (PBS), and then incubated with 100 μ g of recombinant gB2 (lot 10 t CHAR) for 10 minutes. The gB2-DOTAP mixture was diluted to a volume of 5 mL with PBS, and immediately applied to a monolayer of 70-80% confluent Class I MHC compatible MC57 cells, or MHC incompatible SVBalb fibroblasts in a 60 mm tissue culture dish. After an overnight incubation, cells were washed and labelled with $\text{Na}^{51}\text{CrO}_4$ for 90 minutes. Control target cells were ^{51}Cr -labelled MC57 or SVBalb fibroblasts that were untreated, or pulsed with a 15 amino acid peptide (TSSIEFARLQFTYNH) that was previously demonstrated to represent a CTL epitope within the gB2 protein. As shown in Table 1, histocompatible MC57 target cells pulsed with the 15mer peptide were killed by the HSV-specific CTL from infected mice. This result was expected, as short peptides associate with Class I MHC molecules on the outside of the cell, and thus do not have to gain access to the interior of the cell for sensitization to occur. In contrast target cells treated with whole gB2 were not efficiently lysed unless DOTAP was present during the overnight incubation. Histoincompatible SVBalb target cells pulsed with the 15 amino acid peptide or DOTAP-gB2 were not lysed, indicating that the effector cells were Class I MHC restricted CTL. A dose response analysis revealed that as little as 1 μ g of recombinant gB2 was sufficient to sensitize target cells for lysis by HSV-specific CTL. Brefeldin A, a drug that blocks processing and presentation of antigen by Class I MHC molecules, prevented sensitization of MC57 by DOTAP-gB2 when included in the culture medium. However, when the 15 amino acid peptide was used to pulse these target cells lysis was observed. Taken together, these results strongly indicate that DOTAP delivered the gB2 protein into the cytoplasm of the target cell, where it was processed for presentation by Class I MHC molecules (Table 2).

TABLE 1. DOTAP modified gB2 sensitizes target cells for lysis by HSV specific CTL^a.

5	Target ratio: Cell	Antigen	% ⁵¹ Cr release from targets at E:T		
			50:1	10:1	2:1
10	MC57 ^b	NONE	< 1 ^c	< 1	< 1
		gB ₂ peptide	78	55	29
		gB ₂	9	2	4
		gB ₂ + DOTAP	41	30	21
15	SVBalb	gB ₂ Peptide	14	8	3
		gB ₂	8	2	< 1
		gB ₂ + DOTAP	3	< 1	5

20 ^a C57B1/6 mice were infected subcutaneously with 1×10^6 pfu of HSV. Five days later draining lymph node cells were cultured for 3 days, and then used as a source of anti-HSV CTL.

25 ^b MC57 (histocompatible) or SVBalb (histoincompatible) target cells were incubated overnight with gB₂ in the presence or absence of DOTAP. DOTAP (30 μ L) was diluted to 100 μ L with PBS, then mixed for 10 minutes with 100 μ g of gB₂. The DOTAP-gB₂ mixture was then diluted to 5 mL with PBS, and added immediately to 70-80% confluent monolayers of target cells. Target cells were washed and
30 labelled for 90 min with Na₂⁵¹CrO₂, washed and then cocultured with HSV-specific CTL at various effector:target cell ratios.

^c Values represent per cent specific ⁵¹Cr release.

35

TABLE 2. Brefeldin A blocks target cell Sensitization by DOTAP-gB₂.

5	gB ₂ peptide	gB ₂ ^a	DOTAP	BFA ^b	% ⁵¹ Cr release from MC57 targets:		
					50:1 ^c	10:1	2:1
10	---	---	---	---	10	<1	<1
	+	---	---	---	57	30	8
	---	+	---	---	14	<1	<1
	---	+	+	---	49	46	25
15	---	+	+	+	14	11	6
	+	+	+	+	68	48	34
20	^a MC57 target cells were treated with 5 µg of gB ₂ in the presence or absence of DOTAP as described in the legend to Table 1. gB ₂ peptide was added at the time of ⁵¹ Cr labelling.						
25	^b Brefeldin A was added to MC57 cells at the same time as DOTAP-gB ₂ .						
	^c Killer:target cell ratio						
	^d Values represent % specific ⁵¹ Cr release in a 4 hr assay.						

30

(B) The ability of DOTAP to deliver gB₂ to antigen presenting cells for reactivation and expansion of HSV-immune lymphocytes from infected mice was studied. Peritoneal exudate cells (PEC) from C57Bl/6 mice were treated with DOTAP and 100 µg of gB₂ overnight. Cells (3×10^6) were then washed and incubated with 3×10^7 spleen cells from C57Bl/6 mice infected 4 weeks earlier with HSV. After five days, the cultured cells were tested for their ability to lyse target cells pulsed with the 15 amino acid gB₂ peptide. As shown in Table 3, the effector cells from this culture lysed the peptide pulsed syngeneic MC57 cells, but not the allogeneic SVBalb targets.

TABLE 3. DOTAP-gB₂ treated macrophages restimulate HSV-specific CTL activity from immune cells^a.

5	Target Cell	gB ₂ peptide	% ⁵¹ Cr release from targets at E:T ratio:		2:1
			50:1	10:1	
	MC57	—	8 ^c	3	<1
		+	85	72	32
10	5VBalb	+	14	5	2

15 ^a Peritoneal exudate cells were induced in C57B1/6 mice by i.p. thioglycollate injection. Cells were harvested and treated overnight with DOTAP and 100 µg of gB₂ as described in the legend to Table 1. Cells (3 × 10⁶) were then cultured for 5 days with 3 × 10⁷ spleen cells from C57B1/6 mice infected 4 weeks previously with 10⁶ pfu of HSV.

20 ^b Killer:Target cell ratio.

^c Values represent % ⁵¹Cr release in a 4 hr assay.

25 (C) Formulations of the invention were compared with conventional lipid/liposome formulations. Fourteen groups of peritoneal exudate cells (PEC) were prepared as described in part (B) above, and were treated with the following formulations:

- | | | |
|----|-----------|--|
| | Group 1: | untreated control |
| | Group 2: | untreated control, peptide pulsed |
| 30 | Group 3: | gB ₂ (50 µg) + DOTAP (30 µg) |
| | Group 4: | gB ₂ (50 µg) + DOTAP (10 µg) |
| | Group 5: | gB ₂ (50 µg) + DOTAP (3 µg) |
| | Group 6: | inactivated HSV virions (10 ⁵ pfu) |
| | Group 7: | inactivated HSV virions (10 ⁵ pfu) + DOTAP (30 µg) |
| 35 | Group 8: | TransfectACE (17 µg) |
| | Group 9: | TransfectACE (17 µg) + gB ₂ (50 µg) |
| | Group 10: | DPPC/CHOL/DOTAP liposomes (5:4:1) (690 µg) |
| | Group 11: | gB ₂ (50 µg) + DPPC/CHOL/DOTAP liposomes (5:4:1) (690 µg DOTAP) |
| 40 | Group 12: | DPPC/CHOL/stearylamine liposomes (5:4:1) (380 µg SA) |

- Group 13: gB₂ (15 µg) + DPPC/CHOL/SA liposomes (5:4:1)
 Group 14: SVBalb cells + gB₂ 15mer peptide (different class I MHC)

The results (shown in Table 4) demonstrate that formulations of the invention (e.g., groups 3-5 and 7) induce significant cell-mediated immunity. In contrast, formulations of SA (stearylamine), DPPC (dipalmitoylphosphatidylcholine) + CHOL (cholesterol) and TransfectACE fail to induce lysis higher than background levels. Note that group 7 demonstrates that the formulations of the invention are capable of inserting intact (inactivated) HSV virions.

TABLE 4: Comparison of different lipids.

	Group	<u>Lysis</u>		
		<u>50:1</u>	<u>12.5:1</u>	<u>3:1</u>
15	1	15	9	3
	2	71	52	28
	3	71	52	24
	4	39	25	11
	5	30	16	11
20	6	14	5	1
	7	30	11	7
	8	11	4	1
	9	14	3	0
	10	8	2	0
25	11	32	15	8
	12	7	1	0
	13	9	4	3
	14	19	14	7

Example 2

(Activity *In vivo*)

The ability of DOTAP modified gB₂ to prime CTL responses in mice was assessed. C57Bl/6 mice were immunized subcutaneously at the base of the tail with 100 µg of gB₂ in the presence of DOTAP or complete Freund's adjuvant, and boosted twice at

weekly intervals with 100 μ g of gB2 and DOTAP or incomplete Freund's adjuvant, respectively. Seven days later, draining lymph nodes were removed and single cell suspensions prepared. Immune cells (3×10^7) were cultured with 1×10^7 syngeneic spleen cells pulsed with the 15 amino acid gB2 peptide. Similar levels of lysis by cultured lymphocytes from the gB2-CFA/IFA immunized animals were observed against untreated and gB2-peptide pulsed MC57 cells (Table 5). However, immune cells from animals immunized with DOTAP gB2 killed gB2 peptide pulsed MC57 cells at levels significantly greater than cells not sensitized with the peptide (Table 5). Lysis was not observed against allogeneic SVBalb cells, indicating that the effector cells were gB2 specific, Class I MHC restricted CTL (Table 5).

TABLE 5. DOTAP modified gB₂ elicits virus-specific CTL responses in mice.

		<u>Per cent specific ⁵¹Cr release from targets:</u>			
15	<u>Adjuvant^a</u>	<u>E:T^b</u> <u>Ratio</u>	<u>MC57</u>		SVBalb
			<u>Untreated</u>	<u>gB₂ peptide</u>	<u>peptide</u>
20	DOTAP	50:1	< 1 ^c	56	4
		10:1	4	29	< 1
		2:1	1	12	< 1
25	CFA/IFA	50:1	9	20	< 1
		10:1	7	12	< 1
		2:1	6	4	< 1

^a C57B1/6 mice were immunized subcutaneously at the base of tail with 100 μ g of recombinant gB₂ and either DOTAP or CFA adjuvant, and were boosted twice at weekly intervals with 100 μ g of gB₂ with DOTAP or IFA, respectively. Seven days later, draining lymph node lymphocytes were cocultured with peritoneal exudate cells that were pulsed with a 15 amino acid gB₂ peptide with the sequence TSSIEFARLQFTYNH previously identified as a CTL epitope. Five days later cultured cells were tested for cytotoxic activity against MHC compatible (MC57) or incompatible (SVBalb) target cells treated with the HSV gB₂ peptide.

^b Killer:target cell ratio.

^c Values represent per cent specific ⁵¹Cr release in a 4 hr assay.

Example 3(Induction of T_C cell activity)

- (A) HIV envelope-specific effector cells (T_C) were generated in Balb/c mice, and tested for cytotoxic activity against target cells sensitized with various forms of recombinant envelope proteins and DOTAP in a 4 hour assay. Targets were treated with 50 μ g of protein and 30 μ g of DOTAP in 5 ml of PBS containing 1 % FCS overnight. gp120 was denatured by treatment with guanidine hydrochloride, 2-mercaptoethanol, and iodoacetamide (Table 6).

TABLE 6: Ability of Various Forms of HIV Envelope to Sensitize Target Cells

Target	Antigen	Form	DOTAP	Percent Specific Lysis at Effector:Target Ratio:		
				50:1	10:1	2:1
SVBalb	None	-	-	10	9	5
	V3HBX2	-	-	17	14	9
	V3SF2	-	-	60	20	15
	gp120	native	+	8	7	5
	gp120	denatured	+	9	9	7
	env 2-3	-	+	45	24	14
MC57	V3SF2	-	-	3	1	1

(B) T_C specific for the V3 loop T_C epitope in gp 120 were generated in Balb/c mice and tested for their ability to lyse histocompatible SVBalb target cells treated with various forms of HIV envelope antigen. Target cells were incubated overnight in 5 ml of PBS containing 1% fetal calf serum, 50 µg of the indicated HIV antigen, and 30 µg of DOTAP. After chromium 51 labelling, cells were incubated with effector cells T_C in a 4 hour assay. gp120 was denatured by treatment with guanidine hydrochloride, 2-mercaptoethanol, and iodoacetamide. Deglycosylation of the denatured gp120 was accomplished by incubating overnight with 100 µg of protein with 1 or 0.1 units of endoglycosidase F and N-glycosidase F (PGNase F) enzymes at 37°C (Table 7).

TABLE 7. Treatment of Target Cells with Deglycosylated gp120 and DOTAP

Target	Antigen	Modification	DOTAP	Percent Specific Lysis:		
				50:1	10:1	2:1
SVBalb	None	none	-	11	6	3
	V3HXB2	none	-	22	13	6
	V3SF2	none	-	78	57	26
	env 2-3	none	+	60	39	20
	gp120	none	+	14	8	4
	gp120	neuraminadase	+	14	7	3
	gp120	denatured	+	14	10	3
	gp120	denatured/PGNaseF (1 unit/100ug)	+	49	41	15
	gp120	denatured/PGNaseF (0.1unit/100ug)	+	45	33	12
MC57	V3SF2		+	14	9	3

- (C) Balb/c mice were immunized subcutaneously with 25 μ g of the indicated HIV envelope protein along with 30 μ g of DOTAP. 3 weeks later, mice were boosted intravenously with 25 μ g of HIV envelope and DOTAP. Spleen cells were restimulated in vitro 3 weeks later with pV3SF2 (env 2-3 and CHO-produced gp120) or pV3HBX2 (baculovirus-produced gp 120_{HB}) and IL-2 for 6 days when cytotoxic activity was assessed in a 4 hour assay against histocompatible (SVBalb) and incompatible (MC57) target cells sensitized with the pV3 peptides (Table 8).

10 TABLE 8. T_C Activity in Mice Immunized with Various Forms of HIV Envelope

Immunization With:			E:T Ratio	Percent Specific Lysis of Target Cells:			
Antigen	DOTAP	SVBalb			MC57		
		None		V3SF2	V3HBX2	V3HBX2	
env 2-3SF2 (yeast)	+	50:1	8	56	14	9	
		10:1	3	35	5	4	
		2:1	2	15	4	1	
gp120IIIb (Baculo)	+	50:1	15	21	73	23	
		10:1	6	11	61	8	
		2:1	3	3	26	3	

- (D) Balb/c mice were infected intraperitoneally with 10^7 pfu of VVgp160 (vaccinia virus expressed gb160), or immunized at -6 and -3 weeks with 25 μ g of CHO-produced gp120 and DOTAP or 100 μ g of pV3SF2 in Complete Freund's Adjuvant subcutaneously. Spleen cells from these mice were restimulated in vitro with pV3SF2 and IL-2 for 7 days when T_C activity was assessed against target cells sensitized with the pV3 peptides in a 4 hour assay (Table 9).

TABLE 9. Immunization of mice with gp120 and DOTAP

Mice Primed With: Immunogen Adjuvant		E:T Ratio	Percent Specific Lysis of Target Cells:			
			SVBalb		MC57	
			None	V3HBX2	V3SF2	V3SF2
VVgp160		50:1	12	13	61	4
		10:1	8	10	34	3
		2:1	6	8	15	2
gp120	DOTAP	50:1	10	21	13	6
		10:1	5	15	7	1
		2:1	1	10	5	1

- (E) C57B1/6 mice were immunized subcutaneously at the base of the tail with 15 μ g of HSV gB mixed with either PBS or MF59. Three weeks later, spleen cells from these mice were cultured with HSV-1 (moi=1) for 5 days, when T_C activity was assessed (Table 10).

5

Table 10. Induction of HSV-gB specific T_C in mice

10	Adjuvant	E:T ^a Ratio	Percent Lysis of MC57 Target Cells Treated With: ^b		
			Untreated	HSV-1	gB(495-509)
10	PBS	40:1	10 ^c	6	9
		10:1	5	0	3
		2.5:1	8	0	3
15	MF59	40:1	13	47	75
		10:1	11	23	31
		2.5:1	5	2	14

a K:T is the killer to target cell ratio in a 4 hour ⁵¹Cr release assay.

20

b Spleen cells were incubated with ⁵¹Cr-labelled MC57 cells that were untreated, infected with HSV-1, or treated with a 15 amino acid peptide gB(495-509) which contains a T_C epitope from the gB protein.

25

c Values represent percent specific ⁵¹Cr release from MC57 target cells.

WHAT IS CLAIMED:

1. A composition for inducing class I MHC-restricted T cell immunity in a mammal or bird, which composition comprises:

- 5 an effective amount of an immunogenic pathogen antigen; and
a lipid suspension comprising a cell membrane-fusible positively-charged lipid in aggregation with said antigen.

2. The composition of claim 1 wherein said lipid is a compound of the
10 formula $R_4\text{-CH(OR}_6\text{)-CH(OR}_5\text{)-N}^+(R_1)(R_2)(R_3) X^\ominus$, wherein
 R_1 , R_2 , R_3 , and R_4 are each independently lower alkyl;
 R_5 and R_6 are each independently lower alkyl, lower alkenyl, or lower alkynyl;
and
 X^\ominus is a non-toxic counterion.

15 3. The composition of claim 1 wherein said lipid suspension consists essentially of DOTAP and aqueous solution.

4. The composition of claim 1, wherein said antigen is characteristic of a
20 pathogen selected from the group consisting of hepatitis A virus, hepatitis B virus, hepatitis C virus, human papilloma virus, herpes simplex virus, human immunodeficiency virus, simian immunodeficiency virus, Epstein-Barr virus, cytomegalovirus, poliovirus, varicella zoster virus, Dengue virus, HTLV-I, HTLV-II, Plasmodium, Chlamydia, pertussis, influenza, diphtheria, tetanus, haemophilus influenza type b, measles, mumps,
25 rubella, cholera, and meningococcus.

5. The composition of claim 4, wherein said antigen is selected from the group consisting of herpes simplex virus proteins.

6. The composition of claim 5, wherein said antigen is selected from the group consisting of HSV-1 gB, HSV-1 gD, HSV-2 gB, and HSV-2 gD.

7. The composition of claim 4, wherein said antigen is selected from the group consisting of cytomegalovirus proteins.

8. The composition of claim 4, wherein said antigen is selected from the group consisting of human immunodeficiency virus proteins.

9. The composition of claim 4, wherein said antigen is selected from the group consisting of hepatitis B virus proteins.

10. The composition of claim 4, wherein said antigen is selected from the group consisting of hepatitis C virus proteins.

11. The composition of claim 4, wherein said antigen is selected from the group consisting of Chlamydia trachomatis proteins.

12. The composition of claim 4, wherein said antigen is selected from the group consisting of pertussis.

13. The composition of claim 4, wherein said antigen is selected from the group consisting of human immunodeficiency virus envelope proteins.

14. The composition of claim 4, wherein said antigen is selected from the group consisting of Epstein-Barr virus proteins.

15. The composition of claim 4, wherein said antigen is selected from the group consisting of varicella zoster virus proteins.

5 16. The composition of claim 4, wherein said antigen is selected from the group consisting of human papilloma virus proteins.

17. The composition of claim 1, wherein said antigen is characteristic of a tumor cell.

10 18. The composition of claim 17, further comprising an effective amount of a cytokine selected from the group consisting of interleukin-2, tumor necrosis factor, and macrophage stimulatory factor.

15 19. A method for inducing class I MHC-restricted T cell immunity in a mammal or bird in need thereof, which method comprises:

administering an effective amount of a composition comprising an effective amount of an immunogenic pathogen antigen, and a cell membrane-fusible lipid suspension comprising DOTAP.

20 20. A method for enhancing class I MHC-restricted cytotoxic T cell immunity in a mammal or bird in need thereof, which method comprises:

administering an effective amount of a composition comprising an effective amount of an immunogenic pathogen antigen; and an adjuvant selected from the group consisting of a submicron emulsion adjuvant and a cell membrane-fusible lipid suspension
25 comprising DOTAP.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/01102

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K9/127; A61K39/00

II. FIELDS SEARCHEDMinimum Documentation Searched⁷

Classification System

Classification Symbols

Int.Cl. 5

A61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸**III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹**

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	US,A,5 049 386 (EPPSTEIN ET AL.) 17 September 1991 see column 38, line 22 - line 33 see column 46 - column 47; example 14 see column 47; example 15 ----	1-20
X	EP,A,0 356 340 (THE LIPOSOME COMPANY, INC.) 28 February 1990 see page 3, line 38 - line 40 see page 3, line 50 - page 4, line 25 -----	1-20

¹⁰ Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

03 JUNE 1993

Date of Mailing of this International Search Report

16.06.93.

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

BENZ K.F.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/01102

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claim 19,20 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9301102
SA 70159

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

03/06/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5049386	17-09-91	US-A- 4897355	30-01-90
		US-A- 4946787	07-08-90
		US-A- 5208036	04-05-93
		AU-B- 594654	15-03-90
		AU-A- 5185386	17-07-86
		EP-A, B 0187702	16-07-86
		JP-A- 61161246	21-07-86

EP-A-0356340	28-02-90	AU-B- 631377	26-11-92
		EP-A- 0356339	28-02-90
		JP-T- 4500203	16-01-92
		WO-A- 9001947	08-03-90
		WO-A- 9001948	08-03-90
